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# Kongeriget Danmark

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**Patent- og Varemærkestyrelsen**  
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Modtaget

**Title:** SPECIFIC AND SENSITIVE DETECTION OF *BACILLUS ANTHRACIS*

**Prior art:**

JP6261760, JP6253846, JP6253847:

5 Polynucleotide for detecting *Bacillus anthracis* and method for detecting *Bacillus anthracis* using the same

US6448016

Nucleotide sequences for detection of *Bacillus anthracis*

US6087104

10 Oligonucleotides for detection of *Bacillus cereus* group bacteria harmful to mammals, and method of detection with the oligonucleotides

US2003082563, EP1304387

Detection of *Bacillus anthracis*

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Single tube nested PCR

- Herrmann et al. J. Clin. Microbiol. (1996) Detection of *Neisseria gonorrhoeae* from Air-Dried Genital Samples by Single-Tube Nested PCR. **34** (10): 2548-2551

20

- Llop et al. Appl. Environ. Microbiol. (2000) Development of a Highly Sensitive Nested-PCR Procedure Using a Single Closed Tube for Detection of *Erwinia amylovora* in Asymptomatic Plant Material. **66** (5):2071-2078

25

- Hurtado et al. Vet. Parasit. (2001) Single tube nested PCR for the detection of *Toxoplasma gondii* in fetal tissues from naturally aborted ewes **102**:17-27

30

- Forsman et al. J. Virol. Methods (2003) Single-tube nested quantitative PCR: a rational and sensitive technique for detection of retroviral DNA. Application to RERV-H/HRV-5 and confirmation of its rabbit origin. **111**:1-11

**Abstract**

5 The invention provides primer sets and probes, as well as kits and methods for the detection of *Bacillus anthracis* in biological or non-biological samples using real-time, single tube nested PCR. The methods of the invention provide for specific and accurate detection of low numbers of *B. anthracis*.

**Description**

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**FIELD OF THE INVENTION**

15 This invention relates to bacterial diagnostics, and more particularly to the detection of *Bacillus anthracis* (anthrax). More specifically, the invention relates to *B. anthracis* specific genetic markers towards which primer sets and hybridization probes have been designed as well as specific single-tube nested amplification of the fragments to be used in a variety of platforms for detection of *B. anthracis*.

## BACKGROUND OF THE INVENTION

*Bacillus anthracis*, the causative agent of Anthrax, is a large, aerobic, Gram-positive, spore-forming, non-motile *Bacillus*. Spores are formed in culture, in the soil, and in the tissues and exudates of dead animals when there is limited access to nutrients, and subsequently not in the blood or tissues of living animals. Spores can remain viable in soil for decades. The bacterium ordinarily produces a zoonotic disease in domesticated and wild animals such as goats, sheep, cattle, horses, and swine. Humans become infected by the cutaneous route (direct contact with diseased animals, industrial work with hides, wool, brushes, or bone meal), by inhalation (Woolsorter's disease), or by ingestion (meat from diseased animals).

Anthrax endospores do not divide, have no measurable metabolism, and are very resistant to drying out, heat, ultraviolet light, gamma radiation, and many disinfectants. All known anthrax virulence genes are expressed by the vegetative form of *B. anthracis* upon germination of spores within the body of the host. Endospores introduced into the body by abrasion, inhalation, or ingestion are phagocytized by macrophages and carried to regional lymph nodes. The endospores germinate inside the macrophages and become vegetative bacteria; the vegetative bacteria are then released from the macrophages, multiply in the lymphatic system, and enter the bloodstream until there is as many as  $10^7$  to  $10^8$  organisms/ml blood, causing massive septicemia. Once they have been released from the macrophages, there is no evidence that an immune response is initiated against vegetative bacilli. Anthrax bacilli express virulence factors, including toxin and capsule polypeptides. The resulting toxemia has systemic effects that lead to the death of the host.

The major virulence factors of *B. anthracis* are encoded on two virulence plasmids, pXO1 (GenBank Accession Nos. AF065404, AE011190, NC001496, and NC003980) and pXO2 (GenBank Accession Nos. AF188935, AE011191, and NC003981). The toxin-bearing plasmid, pXO1, is 181.6 kilobases (kb) in size and comprises the genes that code for the secreted exotoxins. The toxin gene complex is composed of protective antigen (PA), lethal factor (LF), and edema factor (EF). The three exotoxin components combine to form two binary toxins. Edema toxin consists of EF, which is a calmodulin-dependent adenylate cyclase, and PA, the binding moiety that permits entry of the toxin into the host cell. Increased cellular levels of cAMP upset water homeostasis and are believed to be responsible for the massive edema seen in cutaneous anthrax. Edema toxin inhibits neutrophil function in vitro and neutrophil function is impaired in patients with cutaneous anthrax infection. Lethal toxin consists of LF, which is a zinc metalloprotease that inactivates mitogen-activated-protein kinase kinase (MAPKK) in vitro, and PA, which acts as the binding domain. Lethal toxin stimulates the

macrophages to release tumor necrosis factor- $\alpha$  and interleukin- $\beta$ , which are partly responsible for sudden death in systemic anthrax. The capsule-bearing plasmid, pXO2, is 96.2 kb in size and comprises three genes (*capA*, *capB*, and *capC*,) involved in the synthesis of the poly-D-glutamic capsule.

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The exotoxins are thought to inhibit the immune response mounted against infection, whereas the capsule inhibits phagocytosis of vegetative anthrax bacilli. The expression of all known major virulence factors is regulated by host-specific factors such as elevated temperature (>37°C.) and carbon dioxide concentration (>5%) and by the presence of serum components.

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Both plasmids are required for full virulence and the loss of either one result in an attenuated strain. Historically, anthrax vaccines were made by rendering virulent strains free of one or both plasmids. By way of example, *Pasteur* is an avirulent pXO2-carrying strain that is encapsulated but does not express exotoxin components, while *Sterne* is an attenuated strain that carries pXO1 and can synthesize exotoxin components but does not have a capsule. The *Sterne* strain seems to provide the best protection, as this strain comprises the pXO1 plasmid and thus is able to synthesize the Protective Antigen, a protein that gives the optimum immunogenic response in vaccines.

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### Genes Encoding Toxins

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#### Protective Antigen (PA)

PA (SwissProt Accession No. P13423; GenBank Accession No. M22589) is the most extensively characterized component of anthrax toxin. The gene for PA is encoded at the *pag* locus on the plasmid pXO1. The gene has been cloned and sequenced and found to contain a 2319 basepair (bp) open reading frame of which 2205 bp encode an A/T-rich (69%) cysteine-free, 735 amino acid (83 kilodalton (kDa)) secreted protein. Although PA is a component of anthrax toxin, it is not toxic by itself.

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The protective antigen (PA) binds to surface receptors on the host cells and is subsequently cleaved by a host cellular protease. The larger C-terminal piece of PA remains bound to the receptor and then binds either EF or LF, which enters the cell by endocytosis. Cleavage occurs at the consensus sequence RKKR, resulting in the removal of a 20-kDa fragment, and yielding PA. Deletions of or mutations at this cleavage site render PA resistant to proteolysis and consequently non-toxic in combination with LF or EF. Removal of the NH<sub>2</sub>-terminal fragment is apparently necessary to expose a region of PA that can bind to the other toxin components, since un-cleaved PA can bind to a cell but is unable to associate with EF or LF.

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### **Lethal Factor (LF)**

LF (SwissProt Accession No. P15917; GenBank Accession Nos. M29081 and M30210), is encoded on the pXO1 plasmid. The gene called Lef has been cloned and sequenced, and found to contain a 2427 bp open reading frame encoding a 33 amino acid signal peptide and the 776 amino acid (90.2 kDa) mature, secreted protein. Mitogen-activated protein kinase (MAPK) kinases 1 and 2 (MAPKK1, MAPKK2) act as substrates for LF.

### **Edema Factor (EF)**

EF (SwissProt Accession No. P40136; GenBank Accession No. M24074), is encoded at the cya locus on the pXO1 plasmid. The gene has been cloned and sequenced and found to contain a 2400-bp-long open reading frame encoding a 33 amino acid signal sequence and a 767 amino acid (88.8 kDa) mature secreted protein. The NH<sub>2</sub>-terminal residues 1-250 share homology with the corresponding region of LF and has been demonstrated to mediate binding to PA.

## **Routes of Infection**

### ***Cutaneous Anthrax***

Cutaneous anthrax accounts for more than 95% of the anthrax cases. After inoculation, the incubation period is 1-5 days. Within a few days, a small papule emerges that becomes vesicular. The latter is filled with blue-black edema fluid – often referred to as a malignant pustule. The vesicle ruptures, leaving a necrotic ulcer. The lesion usually is painless, and varying degrees of edema may be present around it. The ulcer develops a characteristic black eschar. (Giving the anthrax its name, anthrakos = coal in Greek) After a period of 2-3 weeks, the eschar separates, often leaving a scar. The mortality rate should be less than 1% with adequate treatment. There are a few case reports of transmissions by insect bites, presumably after the insect fed on infected carcasses.

### ***Gastrointestinal Anthrax***

Gastrointestinal anthrax results from the ingestion of infected meat from diseased animals. Ingested *B. anthracis* proliferate within the gastrointestinal tract, invades the epithelium and ulcerates the mucosa. The invasion spreads to the lymph nodes and then to the bloodstream. After an incubation period of 2-5 days there is vomiting and diarrhea followed by blood in the feces. However, there are reports of both constipation and diarrhea. Invasion of the bloodstream is associated with profound fatigue, shock and death. Mortality rate may be as high as 50%.

### ***Inhalational Anthrax***

Pulmonary / inhalation anthrax (also known as woolsorter's disease) results from inhalation of *B. anthracis* spores between 2 and 5  $\mu\text{m}$  in size. A size that is optimal for inhalation and deposition in the alveolar spaces at which they are phagocytized by the alveolar macrophages in which they subsequently germinate and replicate. The minimum infectious inhaled dose in primates is 4,000 to 10,000 spores. Although the minimum infectious inhaled dose in humans has not yet been determined, the US Department of Defense estimates that the LD<sub>50</sub> (lethal dose for 50% of test subjects) for humans is between 8,000 and 10,000 spores. The typical incubation period is 1-6 days, but a latent period as long as 60 days has been described. The injured host cell and organisms infect the lymph nodes where marked hemorrhagic necrosis may occur. Initial manifestations are non-specific and include fever, malaise, and a non-productive cough. Once in the lymph nodes, infection may spread into the blood stream. Respiratory distress and cyanosis are manifestations of toxemia. Death results within 24 hours. This form of anthrax is of significance in biological warfare.



## SUMMARY

The invention provides for methods of a sensitive and highly specific identification of *Bacillus anthracis* in a biological sample or in a non-biological sample. Primers and probes for detecting  
5 *B. anthracis* are provided by the invention, as are kits containing such primers and probes. Methods of the invention can be used to rapidly identify *B. anthracis* DNA from specimens for diagnosis of *B. anthracis* infection and to identify hoax cases of *B. anthracis*. Using specific primers and probes, the methods include amplifying and monitoring the development of specific amplification products using detection systems based on either voltametric analysis of  
10 electrochemically active probes or detection of fluorescence.

In one aspect of the invention, a method is provided for detecting the presence or absence of *B. anthracis* in a biological sample from an individual or in a non-biological sample. Using a polymerase having additional 5'-3' exonuclease activity, the method to detect *B. anthracis*  
15 includes performing at least one cycling step, which includes an amplifying step and a hybridizing step. The amplifying step includes contacting the sample with a pair of capA primers to produce a capA amplification product if a *B. anthracis* capA nucleic acid molecule is present in the sample.

20 In another aspect of the invention, there is provided a method for further amplifying the previously capA amplification product in the same reaction by contacting said capA amplification product with another pair of nested capA primers to generate another capA amplification product if a *B. anthracis* capA nucleic acid molecule is present in the sample.

25 Functional isolation of the two capA primer pairs is achieved by designing primer pairs with different  $T_m$  values and by using different molar amounts of outer and nested capA primers, the molar ratio being between 1/10 and 1/500. The outer capA primers are designed to have a  $T_m$  10°C higher than the nested capA primers. The probe is designed to have a  $T_m$  10°C higher than the  $T_m$  of the nested capA primers, and to hybridize as little as possible with any of the  
30 capA primers, nor with copies of itself.

The hybridizing step includes contacting the sample with a capA probe. The capA probe is typically labeled with an electrochemically active marker such as a metallocene, more specifically ferrocene. In solution, the accumulated digested probe will be distinguished from  
35 undigested probe due to its different electrochemical activity; the method therefore further includes the detection of the presence or absence of a probe-specific voltage peak using a detection system based on voltametric analysis of electrochemical activity. The presence of a

probe-specific voltage peak is usually indicative of the presence of *B. anthracis* in the sample, while the absence of a probe-specific voltage peak is usually indicative of the absence of *B. anthracis* in the sample.

5 Alternatively, the capA probe is labeled at the most 5' base with a fluorescent dye and at the most 3' base with a fluorescent quencher dye which hybridizes within the target DNA; said labeled oligonucleotide probe being susceptible to 5'-3' exonuclease degradation by said polymerase to produce fragments that can be detected by fluorogenic methods. The presence of fluorescence is usually indicative of the presence of *B. anthracis* in the sample, while the  
10 absence of fluorescence is usually indicative of the absence of *B. anthracis* in the sample.

Alternatively or additionally, the amplifying step can include contacting the sample with a pair of Lef primers to produce an amplification product if a *B. anthracis* Lef nucleic acid molecule is present in the sample.

15 In another aspect of the invention, there is provided a method for further amplifying the previously Lef amplification product in the same reaction by contacting said Lef amplification product with another pair of nested Lef primers to generate another Lef amplification product if a *B. anthracis* Lef nucleic acid molecule is present in the sample.

20 Functional isolation of the two Lef primer pairs is achieved by designing primer pairs with different  $T_m$  values and by using different molar amounts of outer and nested Lef primers, the molar ratio being between 1/10 and 1/500. The outer Lef primers are designed to have a  $T_m$  10°C higher than the nested Lef primers. The probe is designed to have a  $T_m$  10°C higher than  
25 the  $T_m$  of the nested Lef primers, and to hybridize as little as possible with any of the Lef primers, nor with copies of itself.

The hybridizing step includes contacting the sample with a Lef probe. The Lef probe is typically labeled with an electrochemically active marker such as a metallocene, more specifically  
30 ferrocene. In solution, the accumulated digested probe will be distinguished from undigested probe due to its different electrochemical activity; the method therefore further includes the detection of the presence or absence of a probe-specific voltage peak using a detection system based on voltametric analysis of electrochemical activity. The presence of a probe-specific voltage peak is usually indicative of the presence of *B. anthracis* in the sample, while the  
35 absence of a probe-specific voltage peak is usually indicative of the absence of *B. anthracis* in the sample.

Alternatively, the Lef probe is labeled at the most 5' base with a fluorescent dye and at the most 3' base with a fluorescent quencher dye which hybridizes within the target DNA; said labeled oligonucleotide probe being susceptible to 5'-3' exonuclease degradation by said polymerase to produce fragments that can be detected by fluorogenic methods. The presence of fluorescence is usually indicative of the presence of *B. anthracis* in the sample, while the absence of fluorescence is usually indicative of the absence of *B. anthracis* in the sample. The methods to detect *B. anthracis* using capA and/or Lef can be performed individually, sequentially or simultaneously.

- 10 Suitable electrochemically active markers include those comprising metallo-carbocyclic pi complexes, that is organic complexes with partially or fully delocalized pi electrons. Suitable markers include those comprising sandwich compounds, in which two carbocyclic rings are parallel, and also bent sandwiches (angular compounds) and monocyclopentadienyls.
- 15 Preferably, the electrochemically active markers are metallocenyl labels. More preferably they are ferrocenyl labels. A representative label for the probe is ferrocenyl and metallocenyl, more advantageously N-substituted ferrocene or metallocene carboxamides. The ferrocene or metallocene ring, which constitutes the labeling moiety, may be un-substituted. Additional corresponding suitable electrochemically active markers are known in the art.

20 In one aspect, the detecting step includes differential pulse voltammetry. The voltammogram traces for the two markers should have probe-specific voltage peaks that are resolvable from each other. In another aspect, the detecting step includes quantitating the probe-specific voltage peaks. In yet another aspect, the detecting step can be performed after each cycling step (e.g., in real-time).

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In yet another aspect, the detecting step includes exciting the sample at a wavelength absorbed by the fluorescent moiety and visualizing and/or measuring the wavelength emitted. In another aspect, the detecting step includes quantitating the fluorescence. In yet another aspect, the detecting step can be performed after each cycling step (e.g., in real-time).

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Generally, the presence of probe-specific voltage peaks (as measured by differential pulse voltammetry) within 45 cycles (e.g., 20, 25, 30, 35, or 40 cycles) indicates the presence of a *B. anthracis* infection in the individual. In addition, determining the melting temperature between the capA probe and the capA amplification product or, similarly, between the Lef probe and the Lef amplification product, respectively, can confirm the presence or absence of the *B. anthracis*.

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Alternatively or additionally, the presence of fluorescence within 45 cycles (e.g., 20, 25, 30, 35, or 40 cycles) indicates the presence of a *B. anthracis* infection in the individual.

5 Representative biological sample include dermal swabs, cerebrospinal fluid, blood, sputum, bronchio-alveolar lavage, bronchial aspirates, lung tissue, and urine. Non-biological samples include powders, air samples, surface swipes, and rinse products from solid materials. Biological or non-biological samples can be cultured. The culture then can be evaluated for *B. anthracis* using the methods of the invention.

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In addition, the cycling step can be performed on a control sample. A control sample can include the same portion of the *B. anthracis* capA nucleic acid molecule. Alternatively, a control sample can include a nucleic acid molecule other than a *B. anthracis* capA nucleic acid molecule. Cycling steps can be performed on such a control sample using a pair of control  
15 primers and a control probe. The control primers and probe are other than capA primers and capA probe. One or more amplifying steps produce a control amplification product. Each of the control probes hybridizes to the control amplification product.

In another aspect of the invention, there are provided articles of manufacture, or kits. Kits of  
20 the invention can include a pair of capA primers, and a capA probe, and a donor and corresponding acceptor fluorescent moieties. For example, the first capA primer provided in a kit of the invention can have the sequence 5'-GGC GAA ACA TGA CGA AAA AC-3' (SEQ ID NO:1) and the second capA primer can have the sequence 5'-CCT CGT TAT GTA GCA ATC GTA TTA C-3' (SEQ ID NO:2). The capA probe provided in a kit of the invention can have the  
25 sequence 5'-CCA TCG TCA TCG TCA AT-3' (SEQ ID NO:3).

For example, the first nested capA primer provided in a kit of the invention can have the sequence 5'-TTA CGT GAC GTC CCA TC-3' (SEQ ID NO:1) and the second nested capA primer can have the sequence 5'-TGC GAC ATG GGT ACA AC-3' (SEQ ID NO:2).

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Articles of manufacture of the invention can further or alternatively include a pair of Lef primers, a pair of page probes, and a donor and corresponding acceptor fluorescent moieties. For example, the first Lef primer provided in a kit of the invention can have the sequence 5'-AAA AGG TAA CAA ATT ACT TAG TTG ATG G-3' (SEQ ID NO:5), and the second Lef primer can  
35 have the sequence 5'-CGA AGT TAA ATT ACT CCC TTC TTC CTT-3' (SEQ ID NO:6). The Lef probe provided in a kit of the invention can have the sequence 5'-TCA AAA GGT GTA GAA TTA AGG-3' (SEQ ID NO:7).

For example, the first nested Lef primer provided in a kit of the invention can have the sequence 5'- GGG TTA TAT GTT CCA GAA TC-3' (SEQ ID NO:1) and the second nested Lef primer can have the sequence 5'-GTA ACT AAA TCA GAT TGG TTC T-3' (SEQ ID NO:2).

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The article of manufacture can include a package insert having instructions thereon for using the primers, and probes to detect the presence or absence of *B. anthracis* in a sample.

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In yet another aspect of the invention there is provided a method for detecting the presence or absence of *B. anthracis* in a biological sample from an individual or in a non-biological sample.

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Such a method includes performing at least one cycling step. A cycling step can include an amplifying step and a hybridizing step. Generally, an amplifying step includes contacting the sample with a pair of capA primers to produce a capA amplification product if a *B. anthracis* capA nucleic acid molecule is present in the sample. Generally, a hybridizing step includes contacting the sample with a capA probe. Such a capA probe is usually labeled with a fluorescent dye and with a fluorescent quencher dye. The method further includes detecting the presence or absence of fluorescence. The presence or absence of fluorescence is indicative of the presence or absence of *B. anthracis* in said sample. In addition to the capA primers/probe described herein, this method also can be performed using Lef and/or Lef

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primers/probe.

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In another aspect of the invention, there is provided a method for detecting the presence or absence of *B. anthracis* in a biological sample from an individual or in a non-biological sample. Such a method includes performing at least one cycling step. A cycling step can include an amplifying step and a dye-binding step. An amplifying step generally includes contacting the sample with a pair of capA primers to produce a capA amplification product if a *B. anthracis* capA nucleic acid molecule is present in the sample. A dye-binding step generally includes contacting the capA amplification product with a double-stranded DNA binding dye. The method further includes detecting the presence or absence of binding of the double-stranded

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DNA binding dye into the amplification product. According to the invention, the presence of binding is typically indicative of the presence of *B. anthracis* in the sample, and the absence of binding is typically indicative of the absence of *B. anthracis* in the sample. Such a method can further include the steps of determining the melting temperature between the capA amplification product and the double-stranded DNA binding dye. Generally, the melting temperature confirms the presence or absence of *B. anthracis*. Representative double-stranded DNA binding dyes include SYBRGreen™, SYBRGold™, and ethidium bromide.

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Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs.

Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, suitable methods and materials are described

5 below. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including definitions, will control.

10 The details of one or more embodiments of the invention are set forth in the accompanying drawings and the description below. Other features, objects, and advantages of the invention will be apparent from the drawings and detailed description, and from the claims.

## DETAILED DESCRIPTION

A real-time assay for detecting *B. anthracis* in a biological sample or in a non-biological sample that is more sensitive and specific than existing assays is described herein. Primers and probes for detecting *B. anthracis* infections and articles of manufacture containing such primers and probes are provided by the invention. The increased sensitivity of real-time PCR for detection of *B. anthracis* compared to other methods, as well as the improved features of real-time PCR including sample containment and real-time detection of the amplified product, make feasible the implementation of this technology for routine diagnosis of *B. anthracis* infections in the clinical laboratory.

### *B. anthracis* Nucleic Acids and Oligonucleotides

The invention provides methods to detect *B. anthracis* by amplifying, for example, a portion of the *B. anthracis* *capA*, or *Lef* nucleic acid. *B. anthracis* nucleic acids other than those exemplified herein (e.g., other than *capA* or *lef*) also can be used to detect *B. anthracis* in a sample and are known to those of skill in the art. The nucleic acid sequence of *B. anthracis* *capA* (encoding encapsulation protein A) and *Lef* (encoding lethal factor) are available (see, for example, GenBank Accession Nos. M24150, M29081, and M30210). Specifically, primers and probes to amplify and detect *B. anthracis* *capA* nucleic acid molecules are provided by the invention, as are primers and probes to amplify and detect *B. anthracis* *Lef* nucleic acid molecules and *B. anthracis* *Lef* nucleic acid molecules.

Primers that amplify a *B. anthracis* nucleic acid molecule, e.g., *B. anthracis* *capA* or *Lef* can be designed using, for example, a computer program such as VectorNTI™ (Informax, Inc., Frederick, Maryland.). Important features when designing oligonucleotides to be used as amplification primers include, but are not limited to, an appropriate size amplification product to facilitate detection (e.g., by electrophoresis), similar melting temperatures for the members of a pair of primers, and the length of each primer (i.e., the primers need to be long enough to anneal with sequence-specificity and to initiate synthesis but not so long that fidelity is reduced during oligonucleotide synthesis). Typically, oligonucleotide primers are 15 to 30 nucleotides in length.

Designing oligonucleotides to be used as hybridization probes can be performed in a manner similar to the design of primers. In addition, probes can be designed to hybridize to targets that contain a polymorphism or mutation, thereby allowing differential detection of *B. anthracis* strains based on either absolute hybridization of different pairs of probes corresponding to the particular *B. anthracis* strain to be distinguished or differential melting temperatures between,

for example, members of a pair of probes and each amplification product corresponding to a *B. anthracis* strain to be distinguished. As with oligonucleotide primers, oligonucleotide probes usually have similar melting temperatures, and the length of each probe must be sufficient for sequence-specific hybridization to occur but not so long that fidelity is reduced during synthesis. Oligonucleotide probes are generally 15 to 30 nucleotides in length.

#### **Polymerase chain reaction (PCR)**

U.S. Pat. Nos. 4,683,202, 4,683,195, 4,800,159, and 4,965,188 disclose conventional PCR techniques. PCR typically employs two oligonucleotide primers that bind to a selected nucleic acid template (e.g., DNA or RNA). Primers useful in the present invention include oligonucleotides capable of acting as a point of initiation of nucleic acid synthesis within *B. anthracis* capA or Lef. A primer can be purified from a restriction digest by conventional methods, or it can be produced synthetically. The primer is preferably single-stranded for maximum efficiency in amplification, but the primer can be double-stranded. Double-stranded primers are first denatured, i.e., treated to separate the strands. One method of denaturing double stranded nucleic acids is by heating.

The term "thermo stable polymerase" refers to a polymerase enzyme that is heat stable, i.e., the enzyme catalyzes the formation of primer extension products complementary to a template and does not irreversibly denature when subjected to the elevated temperatures for the time necessary to effect denaturation of double-stranded template nucleic acids. Generally, the synthesis is initiated at the 3' end of each primer and proceeds in the 5' to 3' direction along the template strand. Thermo stable polymerases have been isolated from *Thermus flavus*, *T. ruber*, *T. thermophilus*, *T. aquaticus*, *T. lacteus*, *T. rubens*, *Bacillus stearothermophilus* and *Methanothermus fervidus*. Nonetheless, polymerases that are not thermo stable also can be employed in PCR assays provided the enzyme is replenished.

If the *B. anthracis* template nucleic acid is double-stranded, it is necessary to separate the two strands before it can be used as a template in PCR. Strand separation can be accomplished by any suitable denaturing method including physical, chemical or enzymatic means. One method of separating the nucleic acid strands involves heating the nucleic acid until it is predominately denatured (e.g., greater than 50%, 60%, 70%, 80%, 90% or 95% denatured). The heating conditions necessary for denaturing template nucleic acid will depend, e.g., on the buffer salt concentration and the length and nucleotide composition of the nucleic acids being denatured, but typically range from about 90°C to about 105°C for a time depending on features of the reaction such as temperature and the nucleic acid length. Denaturation is typically performed from about 2 seconds (in lab-on-chip settings) to 10 minutes.



After the double-stranded nucleic acid is denatured by heat, the reaction mixture is allowed to cool to a temperature that promotes annealing of each primer to its target sequence on the *B. anthracis* nucleic acid. The temperature for annealing is usually from about 35°C to about 65°C. Annealing times can be from about 1 second (in lab-on-chip settings) to about 1 min. The reaction mixture is then adjusted to a temperature at which the activity of the polymerase is promoted or optimized, i.e., a temperature sufficient for extension to occur from the annealed primer to generate products complementary to the template nucleic acid. The temperature should be sufficient to synthesize an extension product from each primer that is annealed to a nucleic acid template, but should not be so high as to denature an extension product from its complementary template (e.g., the temperature for extension generally ranges from about 40°C to 80°C.). Extension times can be from about 5 seconds (in lab-on-chip settings) to about 5 minutes.

PCR assays can employ *B. anthracis* nucleic acid such as DNA or RNA, including messenger RNA (mRNA). The template nucleic acid need not be purified; it may be a minor fraction of a complex mixture, such as *B. anthracis* nucleic acid contained in human cells. DNA or RNA may be extracted from a biological sample such as dermal swabs, cerebrospinal fluid, blood, sputum, bronchio-alveolar lavage, bronchial aspirates, lung tissue, and feces by routine techniques employed by persons known in the art. If *B. anthracis* is present, DNA or RNA also can be extracted from non-biological samples such as air samples, suspicious powders, surface swipes, and rinse products from suspicious solid materials. Nucleic acids can be obtained from any number of sources, such as plasmids, or natural sources including bacteria, yeast, viruses, organelles, or higher organisms such as plants or animals.

The oligonucleotide primers are combined with PCR reagents under reaction conditions that induce primer extension. For example, chain extension reactions generally include 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 15 mM MgCl<sub>2</sub>, 0.5-1.0 µg denatured template DNA, 50 pmoles of each oligonucleotide primer, 2.5 U of Taq polymerase, and 10% DMSO). The reactions usually contain 150 to 300 µM each of dATP, dCTP, dTTP, dGTP, or one or more analogs thereof.

The newly synthesized strands form a double-stranded molecule that can be used in the succeeding steps of the reaction. The steps of strand separation, annealing, and elongation can be repeated as often as needed to produce the desired quantity of amplification products corresponding to the target *B. anthracis* nucleic acid molecule. The limiting factors in the reaction are the amounts of primers, thermo stable enzyme, and nucleoside triphosphates present in the reaction. The cycling steps (i.e., denaturation, annealing, and extension) are

preferably repeated at least once. For use in detection, the number of cycling steps will depend, e.g., on the nature of the sample. If the sample is a complex mixture of nucleic acids, more cycling steps will be required to amplify the target sequence sufficient for detection. Generally, the cycling steps are repeated at least about 20 times, but may be repeated as many as 40, 60, or even 100 times.

#### **Differential pulse voltammetry (DPV)**

DPV technology (see, for example, U.S. Pat. No. 4,083,754 ) is used for both qualitative and quantitative analysis. The method take advantage of computer timing to repeatedly sample current signals at two points relative to the time of application of a voltage signal to the electrode. The difference between the two current values is plotted as a function of the applied DC potential. The resultant is peaks, corresponding to the electro-activity of the species in the electrochemical cell.

Electrochemical methods, when compared to the conventional methods for DNA analysis (e.g., fluorescence), are straightforward and sensitive and do not require sophisticated instrumentation. Consequently, they are suitable for the development of inexpensive and portable devices for detection and disease diagnoses. Typically, the DNA hybridization is detected by electrochemical reactions of a redox marker or reporter on the target (e.g., ferrocene).

#### **Detection of *B. anthracis***

The presence of *B. anthracis* is generally being detected by culturing the organism. The success of culturing *B. anthracis* from clinical specimens depends in part upon the *B. anthracis* infection and hence, the source of the specimen. Cultures from skin lesions associated with the cutaneous form of the disease can exhibit 60-65% sensitivity, and often are not diagnostically useful. Generally, cultures from blood exhibit a high sensitivity due to the extremely large number of circulating *B. anthracis* organisms. Patients with systemic disease, however, often expire within the time necessary for blood cultures to become positive. Other biological samples including sputum and cerebrospinal fluid (CSF) can also be used for culture,, but the identification may come too late to initiate effective antibiotic therapy. In the case of gastrointestinal anthrax, cultures from stool samples also can be used.

In addition, serologic tests including enzyme-linked immunosorbent assays (ELISAs) for the detection of *B. anthracis* have been reported. The sensitivity of ELISA to detect serum antibodies to various targets in *B. anthracis* including the encapsulation protein, protective

antigen, lethal factor, and edema factors varies between 26% and 100%, depending upon the target and study.

5 Direct detection of *B. anthracis* from clinical specimens or suspicious substances using stains is possible and can allow a presumptive identification of *B. anthracis*. With Gram staining, the cells are visualized as large Gram-positive encapsulated rods. The success of this staining technique depends upon the presence of a sufficient number of organisms. Typically, staining techniques provide a presumptive identification of *B. anthracis* and a definitive diagnosis generally requires further evaluation.

10 Conventional PCR methods also have been used to detect *B. anthracis*. *B. anthracis* and other members of the *B. cereus* group, however, exhibit a high degree of genomic homology, making detection and differentiation by PCR difficult. Conventional PCR-based amplification is generally followed by transfer of the amplification products to a solid support and detection  
15 using a labeled probe (e.g., a Southern or Northern blot). These methods are labor intensive and frequently require more than one day to complete. Additionally, the manipulation of amplification products for the purpose of detection (e.g., by blotting) increases the risk of carry-over contamination and false positives. By using commercially available real-time PCR instrumentation (e.g., DNA Engine Opticon™ MJ research, Reno, NV.), PCR amplification and  
20 detection of the amplification product can be combined in a single closed cuvette with dramatically reduced cycling time. Since detection occurs concurrently with amplification, the real-time PCR methods prevent the need for manipulation of the amplification product, and therefore diminish the risk of cross-contamination between amplification products. Real-time PCR greatly reduces turn-around time and is an attractive alternative to conventional PCR  
25 techniques in the clinical laboratory.

The present invention provides methods for detecting the presence or absence of *B. anthracis* in a biological sample from an individual or in a non-biological sample. Methods provided by the invention avoid problems of sample contamination, false negatives, and false positives.

30 The methods include performing at least one cycling step that includes amplifying a *B. anthracis* portion of a capA and/or Lef nucleic acid molecule from a sample using a pair of capA and/or Lef primers, respectively. Each of the capA or Lef primers anneals to a target within or adjacent to a *B. anthracis* capA or Lef nucleic acid molecule, respectively, such that at least a portion of each amplification product contains nucleic acid sequence corresponding to capA or  
35 |lef, respectively. More importantly, the amplification product should contain the nucleic acid sequences that are complementary to the capA or Lef probes, respectively. The capA and/or Lef amplification product is produced provided that *B. anthracis* nucleic acid is present. Each

cycling step further includes contacting the sample with a pair of capA and/or Lef probes. According to the invention, the capA and Lef probes are typically labeled with an electrochemically active marker such as a metallocene, more specifically ferrocene. In solution, the accumulated digested probe will be distinguished from undigested probe due to its different electrochemical activity; the method therefore further includes the detection of the presence or absence of a probe-specific voltage peak (PSVP) using a detection system based on voltametric analysis of electrochemical activity. The presence of a probe-specific voltage peak is usually indicative of the presence of *B. anthracis* in the sample, while the absence of a probe-specific voltage peak is usually indicative of the absence of *B. anthracis* in the sample.

Alternatively, the capA and Lef probes are labeled at the most 5' base with a fluorescent dye and at the most 3' base with a fluorescent quencher dye which hybridizes within the target DNA; said labeled oligonucleotide probe being susceptible to 5'-3' exonuclease degradation by said polymerase to produce fragments that can be detected by fluorogenic methods.

Each cycling step includes an amplification step and a hybridization step, and each cycling step is usually followed by respectively a PSVP or FRET detection step. Multiple cycling steps are performed, preferably in a thermocycler. Methods of the invention can be performed using one or more of the capA and/or Lef primer and probe sets to detect the presence of *B. anthracis*.

Alternatively, methods of the invention can be performed simultaneously with each of the capA and Lef primer and probe sets to detect the presence of virulent forms of *B. anthracis*. Detection of PSVP or FRET signal in one or more, but not all, of the capA and Lef reactions indicates the presence of a *B. anthracis* strain lacking one or both of the virulence plasmids. Methods of the invention, therefore, also are useful for detecting false claims of anthrax.

As used herein, "amplifying" refers to the process of synthesizing nucleic acid molecules that are complementary to one or both strands of a template nucleic acid molecule (e.g., *B. anthracis* capA or Lef nucleic acid molecules). Amplifying a nucleic acid molecule typically includes denaturing the template nucleic acid, annealing primers to the template nucleic acid at a temperature that is below the melting temperatures of the primers, and enzymatically elongating from the primers to generate an amplification product. Amplification typically requires the presence of deoxyribonucleoside triphosphates, a DNA polymerase enzyme (e.g., Platinum® Taq) and an appropriate buffer and/or co-factors for optimal activity of the polymerase enzyme (e.g., MgCl<sub>2</sub> and/or KCl).

If amplification of *B. anthracis* nucleic acid occurs and an amplification product is produced, the step of hybridizing results in a detectable signal based upon PSVP or FRET of the degraded

probe. As used herein, "hybridizing" refers to the annealing of probes to an amplification product. Hybridization conditions typically include a temperature that is below the melting temperature of the probes but that avoids non-specific hybridization of the probes.

5 Generally, the presence of PSVP or FRET indicates the presence of *B. anthracis* in the sample, and the absence of PSVP or FRET indicates the absence of *B. anthracis* in the sample. However, inadequate specimen collection, transportation delays, inappropriate transportation conditions, or use of certain collection swabs (calcium alginate or aluminum shaft) are all conditions that can affect the success and/or accuracy of a test result. Using the methods  
10 disclosed herein, detection of PSVP or FRET within 50 nested cycling steps is indicative of a *B. anthracis* infection or contamination.

Representative biological samples that can be used in practicing the methods of the invention include dermal swabs, nasal swaps, cerebrospinal fluid, blood, sputum, bronchio-alveolar  
15 lavage, bronchial aspirates, lung tissue, and feces. Collection and storage methods of biological samples are known to those of skill in the art. Biological samples can be processed (e.g., by nucleic acid extraction methods and/or kits known in the art) to release *B. anthracis* nucleic acid or in some cases, the biological sample can be contacted directly with the PCR reaction components and the appropriate oligonucleotides.

20 Non-biological samples such as air samples (filtered or non-filtered), powders, and surface swipes and rinse products from suspicious materials also can be examined for the detection of *B. anthracis*. For example, a powder can be dissolved in a solvent such as water, and the methods of the invention can be performed on varying dilutions (e.g., 1:10, 1:100, or 1:1000)  
25 of the resulting solution. Water can be added to a collection vial of an air sample collection device and assayed using methods of the invention, or alternatively, a filter on an air sample collection device can be rinsed and assayed. In addition, a solid material (e.g., paper) can be swiped or rinsed for the purpose of detecting *B. anthracis*, and a non-turbid solution produced. Dilutions of such a surface swipe or rinse can be used in a real-time amplification reaction of  
30 the invention.

Biological or non-biological samples can be cultured in a medium suitable for growth of *B. anthracis*. The culture media then can be assayed for the presence or absence of *B. anthracis*  
35 using the methods of the invention as described herein. For example, samples arriving at a clinical laboratory for detection of *B. anthracis* using the methods of the invention can be in the form of a liquid culture that had been inoculated with a biological sample from an individual or with a non-biological sample.

Melting curve analysis is an additional step that can be included in a cycling profile. Melting curve analysis is based on the fact that DNA melts at a characteristic temperature called the melting temperature ( $T_m$ ), which is defined as the temperature at which half of the DNA  
5 duplexes have separated into single strands. The melting temperature of a DNA depends primarily upon its nucleotide composition. Thus, DNA molecules rich in G and C nucleotides have a higher  $T_m$  than those having an abundance of A and T nucleotides. By detecting the temperature at which signal is lost, the melting temperature of probes can be determined. Similarly, by detecting the temperature at which signal is generated, the annealing  
10 temperature of probes can be determined. The melting temperature(s) of the capA or Lef probes from the respective amplification product can confirm the presence or absence of *B. anthracis* in the sample.

Within each thermocycler run, control samples are cycled as well. In the positive control  
15 samples *B. anthracis* nucleic acid control template can be amplified (other than capA or Lef) using, for example, control primers and control probes. Positive control samples can comprise, for example, a plasmid construct containing *B. anthracis* capA or Lef nucleic acid molecule. Such a plasmid control can be amplified internally (e.g., within the sample) or in a separate sample run side-by-side with the samples to be examined. Each thermocycler run should also  
20 include a negative control that, for example, lacks *B. anthracis* template DNA. Such non-template controls are indicators of the success or failure of the amplification, hybridization and/or fluorescence reaction. Therefore, control reactions can readily determine, for example, the ability of primers to anneal with sequence-specificity and to initiate elongation, as well as the ability of the probe to hybridize with sequence-specificity and for fluorescence to occur.

25 Standard laboratory containment practices and procedures are desirable when performing methods of the invention. Containment practices and procedures include, but are not limited to, separate work areas for different steps of a method, containment hoods, barrier filter pipette tips and dedicated air displacement pipettes. Consistent containment practices and  
30 procedures by personnel are necessary for accuracy in a diagnostic laboratory handling clinical samples.

Conventional PCR methods in conjunction with fluorescence technology can be used to practice  
the methods of the invention. In one embodiment, a DNA Engine Opticon™ instrument is used  
35 (a detailed description of the DNA Engine Opticon™ System and real-time and on-line monitoring of PCR can be found at  
<http://www.mjresearch.com/html/instruments/opticon/index.html>).

The DNA Engine Opticon™ can be operated using a PC workstation and can utilize a Windows XP operating system. The software can display the fluorescence signals in real-time immediately after each measurement. Fluorescent acquisition time is 10-100 milliseconds (msec). After each cycling step, a quantitative display of fluorescence vs. cycle number can be continually updated for all samples. The data generated can be stored for further analysis.

An amplification product can be detected using a double-stranded DNA binding dye such as a fluorescent DNA binding dye (e.g., SYBRGreenI® or SYBRGold® (Molecular Probes)). Upon interaction with the double-stranded nucleic acid, such fluorescent DNA binding dyes emit a fluorescence signal after excitation with light at a suitable wavelength. A double-stranded DNA binding dye such as a nucleic acid intercalating dye also can be used. When double-stranded DNA binding dyes are used, a melting curve analysis is usually performed for confirmation of the presence of the amplification product.

As described herein, amplification products can be detected using labeled hybridization probes that take advantage of fluorescence technology. A common format of fluorescence technology utilizes the TaqMan® technology to detect the presence or absence of an amplification product, and hence, the presence or absence of *B. anthracis*. TaqMan® technology utilizes one single-stranded hybridization probe labeled with two fluorescent moieties. When a first fluorescent moiety is excited with light of a suitable wavelength, the absorbed energy is transferred to a second fluorescent moiety. The second fluorescent moiety is generally a quencher molecule. During the annealing step of the PCR reaction, the labeled hybridization probe binds to the target DNA (i.e., the amplification product) and is degraded by the 5' to 3' exonuclease activity of the *Taq* Polymerase during the subsequent elongation phase. As a result, the excited fluorescent moiety and the quencher moiety become spatially separated from one another. As a consequence, upon excitation of the first fluorescent moiety in the absence of the quencher, the fluorescence emission from the first fluorescent moiety can be detected.

Molecular beacons in conjunction with fluorescence also can be used to detect the presence of an amplification product using the real-time PCR methods of the invention. Molecular beacon technology uses a hybridization probe labeled with a first fluorescent moiety and a second fluorescent moiety. The second fluorescent moiety is generally a quencher, and the fluorescent labels are typically located at each end of the probe. Molecular beacon technology uses a probe oligonucleotide having sequences that permit secondary structure formation (e.g., a hairpin). As a result of secondary structure formation within the probe, both fluorescent moieties are in

spatial proximity when the probe is in solution. After hybridization to the target nucleic acids (i.e., amplification products), the secondary structure of the probe is disrupted and the fluorescent moieties become separated from one another such that after excitation with light of a suitable wavelength, the emission of the first fluorescent moiety can be detected.

5

It is understood that the present invention is not limited by the configuration of one or more commercially available instruments.

### Articles of Manufacture

10

The invention further provides for articles of manufacture to detect *B. anthracis*. An article of manufacture according to the present invention can include primers and probes used to detect *B. anthracis*, together with suitable packaging materials. Representative primers and probes for detection of *B. anthracis* are capable of hybridizing to *B. anthracis* capA or Lef nucleic acid molecules. Methods of designing primers and probes are disclosed herein, and representative examples of primers and probes that amplify and hybridize to *B. anthracis* capA or Lef nucleic acid molecules are provided.

15

Articles of manufacture of the invention also can include one or more fluorescent moieties for labeling the probes or, alternatively, the probes supplied with the kit can be labeled. For example, an article of manufacture may include a donor fluorescent moiety for labeling one end of the capA or Lef probes and an acceptor fluorescent moiety for labeling the other end of the capA or Lef probe, respectively. Examples of suitable FRET donor fluorescent moieties and corresponding acceptor fluorescent moieties are provided above.

20

25

Articles of manufacture of the invention also can contain a package insert or package label having instructions thereon for using the capA primers and probes or Lef primers and probes to detect *B. anthracis* in a sample. Articles of manufacture may additionally include reagents for carrying out the methods disclosed herein (e.g., buffers, polymerase enzymes, co-factors, or agents to prevent contamination). Such reagents may be specific for one of the commercially available instruments described herein.

30

The invention will be further described in the following examples, which do not limit the scope of the invention described in the claims.



## EXAMPLES

### Example 1

#### 5 Oligonucleotide Primers and Probes

Primers and probes were designed using the software VectorNTI™ version 5.0 (Informax, Inc., Frederick, Maryland.). Sequences for primers and probes are shown in Table 1. The GenBank Accession numbers for the reference sequences used to design the primers and probes for each target are shown in Table 1, along with the relative location of each primer and probe.

TABLE 1

Primer	Seq ID	Name	Sequence	Length bases	T <sub>m</sub> °C
PX01 primer gives 912 bp product with PXA (for possible use as template)		PXS	5'-AATATCAATAACCTTACAGCAACCC-3'	25	60.0
PX01 primer gives 912 bp product with PXS (for possible use as template)		PXA	5'-ATGCATTAACCTAAAGGCTTCTG-3'	23	59.6
pX02 outer sense primer	1	PcapAinS	5'-GGCGAAACATGACGAAAAAC-3'	20	60.1
pX02 outer antisense primer	2	PcapAinA	5'-CCTCGTTATGTAGCAATCGTATTAC-3'	25	59.4
pX02 inner sense primer	3	PcapAnesS	5'-TTACGTGACGTCCCATC-3'	17	51.2
pX02 inner antisense primer	4	PcapAnesA	5'-TGCGACATGGGTACAAC-3'	17	52.2
PROBE-pX02	5	PcapAprobe	5'- <b>FAM</b> -CAACCATCGTCATCGTCAATT- <b>BHQ</b> -3'	21	60.2
pX01 outer sense primer	6	PXouts	5'-AAAAGGTAACAAATTACTTAGTTGATGG-3'	28	60.5
pX01 outer antisense primer	7	PXouta	5'-CGAAGTTAAATTACTCCCTTCTTCCTT-3'	27	63.4
pX01 inner sense primer	8	PXins	5'-GGGTTATATGTTCCAGAATC-3'	20	50.6
pX01 inner antisense primer	9	PXina	5'-GTAATAAATCAGATTGGTTCT-3'	22	49.9
PROBE-pX01	10	PX-probe	5'- <b>FAM</b> -GACCTTCAAAAGGTGTAGAATTAAGG- <b>BHQ</b> -3'	26	61.2

The pXO2 initial sense and antisense primer set amplifies a 209 base pair region. The pXO1 outer sense and antisense primer set amplifies a 912 base pair region. Primers were adjusted to a stock solution of 100  $\mu$ M.

5

Probes were dissolved in TE-buffer to a concentration of 20  $\mu$ M (supplied with the probes and resuspended according to manufacturer's instructions).

10

The analytical sensitivities for the two gene targets (i.e., *capA* or *lef*) were at least 10 copies of the target sequence.

## Example 2

### PCR Conditions

- 5 The DNA Engine Opticon™ hybridization mixture was identical for each *B. anthracis* gene target (with the exception that each primer and probe set was specific for the particular gene target that was amplified).

- 10 A standard 1x DNA Engine Opticon™ hybridization mixture for *B. anthracis* capA or Lef comprises the following (Karsai et al., BioTechniques 2002, 32(4):790-796):

10 mM TrisHCl, pH 8.5

50 mM KCl

2 mM MgCl<sub>2</sub>

- 15 0.1 % Tween-20 and/or 0.15% Triton X-100

±20 µg/ml of BSA

±0.3% DMSO or 0.8% glycerol

- 20 The DNA Engine Opticon™ thermocycling conditions were identical for each gene target and are listed in the table below:

Number of cycles	Comment	Temperature (°C)	time
1	Denaturation	95.0	5 min
50	Outer primers	95.0	10 sec
		63.0	30 sec
51	Inner primers	95.0	10 sec
		51.0	30 sec
		60.0	30 sec
		4	Until stopped

- 25 To generate the 912 bp pXO1 template, DNA was isolated from an overnight culture of *Bacillus anthracis* grown on solid substrate. Colonies was scraped of, resuspended in TE-buffer and boiled for 15 minutes in sealed PCR tubes using a heated lid thermo cycler. Subsequently, the boiled culture was phenolized and DNA was precipitated and washed with 70% ethanol prior to dissolving in TE-buffer. The purified DNA was used for a PCR reaction involving the pXO1 outer sense and antisense primer set.

The concentration of the template was determined by spectrophotometric fluorescence compared to a standard. The concentration (in µg/ml) was calculated to number of copies using

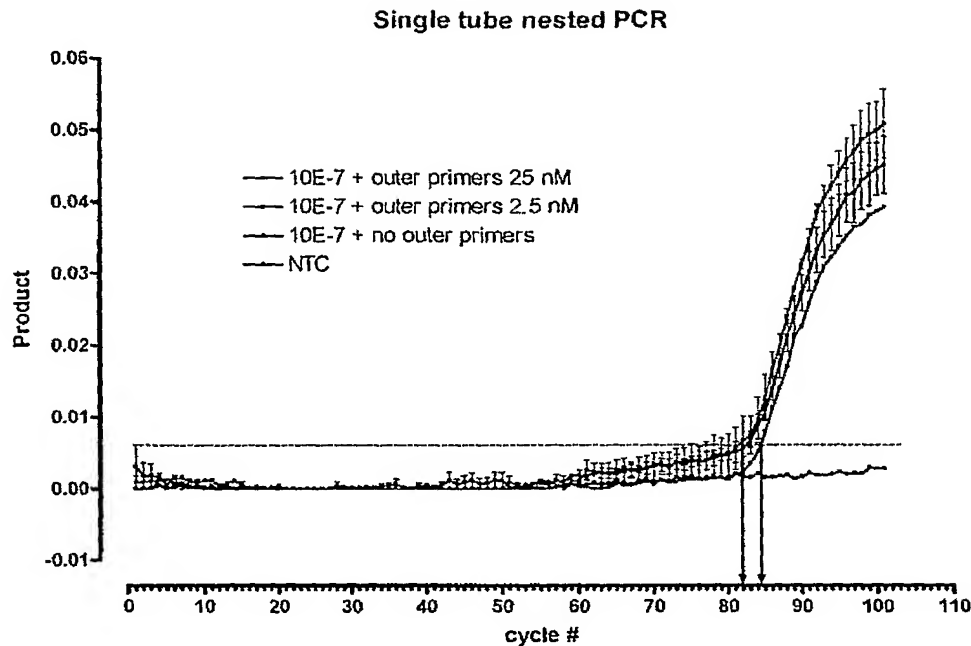
5  $N_A = 6.023 \cdot 10^{23}$

10 
$$\frac{(\%GC \cdot \text{total length})}{100} (618.4) + \frac{(100-\%GC \cdot \text{total length})}{100} (617.4) + 36.0$$

The 912 bp template generated using primers PXA and PXS comprises a G+C content of 30.15% and subsequently an A+T content of 69.85%. Using the above equations, this gives a molecular weight of 563,379 Daltons, i.e., one single molecule weighs  $9.4 \cdot 10^{-19} \text{ g} \approx 1 \text{ ag}$ . The purified 912 bp template stock had a concentration of 1 µg/ml giving the following concentrations and number of molecules in the working solutions:

Dilution	Concentration	Number of molecules per µl	Number of molecules per reaction*
$10^{-3}$	1 pg/µl	$10^6$	$1.67 \cdot 10^5$
$10^{-4}$	0.1 pg/µl	$10^5$	$1.67 \cdot 10^4$
$10^{-5}$	0.01 pg/µl	$10^4$	$1.67 \cdot 10^3$
$10^{-6}$	1 fg/µl	$10^3$	167
$10^{-7}$	0.1 pg/µl	$10^2$	16.7
$10^{-8}$	0.01 pg/µl	10	1.67

\*) Setting up the PCR was done by adding 6 µl of diluted template in 714 µl PCR reaction mixture and running in reaction volumes of 20 µl (i.e., a dilution factor of  $120/20 = 6$ )



Using a working solution of template diluted  $10^{-7}$  (i.e., approximately 17 molecules per reaction), the definite identification of the small number of template molecules was detected in the single tube nested PCR reaction. Furthermore, as evident from the figure, the single tube nested PCR reaction was able to detect the template 3 cycles before the "non-nested" (i.e., no outer primers) reaction – thus exhibiting a 10-fold increase in sensitivity.

### Example 3

#### 10 Melting Curves

Following the completion of the amplification reactions, a melting curve analysis was performed by raising the temperature in the DNA Engine Opticon™ thermal chamber from 50°C to 85°C at 0.2°C per second. Fluorescent measurements were taken continuously as the temperature was raised and melting curves were generated. Each pair of probes had a specific and characteristic melting curve from the respective amplification product. The melting temperatures ( $T_m$ ) for the products generated were  $XX \pm 1^\circ\text{C}$  for capA and  $XX \pm 1^\circ\text{C}$  for Lef.

[determination of melting temperature – to be inserted]

#### **Example 4**

##### Analytical Sensitivity

- 5 A total of three isolates of *B. anthracis* were studied including a strain used for vaccine preparation (ATCC14185). One isolate (SSI2160) was positive for both of the two gene targets. *B. anthracis* 'Pasteur' which lacks plasmid pXO1 was only positive for capA . *B. anthracis* 'Sterne' lacks plasmid pXO2 and was positive for Lef but not for capA .
- The following *B. anthracis* strains were used.

10

Strain	Species	Original code	Source/provider
SSI 2160	<i>B. anthracis</i> virulent	SSI2160 - K670/88	SSI
Sterne	<i>B. anthracis</i> Sterne	ATCC14185	SSI
Pasteur	<i>B. anthracis</i> Pasteur		CIP

SSI: Statens Serum Institute, Denmark

CIP: Collection de l'Institut Pasteur, France

### Example 5

#### Analytical Specificity

- 5 The following organisms were tested essentially as described above in Examples 1 and 2 for cross reactivity with the primers and probes directed toward *B. anthracis*. Instead of using a PCR generated template, chromosomal DNA was extracted from the individual strains using the protocol described by Boe et al. (Boe L, et al. (1989), Replication origins of single-stranded-DNA plasmid pUB110. J Bacteriol 171:3366-3372). There was no cross-reactivity  
10 observed. Some of the organisms listed below were obtained from clinical isolates; others were type strains or environmental isolates. The quality of the purified chromosomal DNA was determined by visual inspection of the preparation after agarose gel electrophoresis and staining. Furthermore, the DNA was used as template for PCR with random primers (RAPD)  
15 and shown to give a product.

The following strains were used:

Strain	Species	Original code	Source/provider/reference
AND 1175	<i>B. cereus</i>		DMU/NERI
AND 1182	<i>B. cereus</i>		Food poisoning – vanilla profiterole
AND 1191	<i>B. cereus</i>	1291	Food poisoning – birthday cake
AND 1204	<i>B. cereus</i>	1552	Food poisoning – boiled potato
AND 1210	<i>B. cereus</i>	1558	Food poisoning – polenta
AND 1213	<i>B. cereus</i>	1561	Food poisoning – uncooked chicken
AND 1228	<i>B. cereus</i>	1576	Food poisoning – uncooked chicken
AND 1232	<i>B. cereus</i>	1584	Food poisoning – boiled rice
AND 1240	<i>B. cereus</i>		Food poisoning – savory crepe
AND 1241	<i>B. cereus</i>	1593	Food poisoning – uncooked chicken
AND 1242	<i>B. cereus</i>	1594	Food poisoning – boiled potato
AND 1258	<i>B. cereus</i>	157/1	food poisoning – boiled rice
GBJ 36	<i>Bt finitimus</i>	4B2 (HD3)	BGSC
GBJ 37	<i>Bt alesti</i>	4C3 (HD4)	BGSC
GBJ 38	<i>Bt kurstaki</i>	4D4 (HD73)	BGSC
GBJ 39	<i>Bt sotto/dendrolimus</i>	4E4 (HD7)	BGSC
GBJ 40	<i>Bt galleriae</i>	4G4 (HD29)	BGSC
GBJ 41	<i>Bt canadensis</i>	4H2 (HD224)	BGSC
GBJ 42	<i>Bt entomocidus/subtoxicus</i>	4I4 (HD9)	BGSC
GBJ 43	<i>Bt aizawai</i>	4J4 (HD11)	BGSC
GBJ 44	<i>Bt morrisoni</i>	4K1 (HD12)	BGSC

GBJ 45	<i>Bt tolworthi</i>	4L3 (HD537)	BGSC
GBJ 46	<i>Bt darmstadensis</i>	4M1 (HD146)	BGSC
GBJ 47	<i>Bt toumanoffi</i>	4N1 (HD201)	BGSC
GBJ 48	<i>Bt thompsoni</i>	4O1 (HD542)	BGSC
GBJ 49	<i>Bt pakistani</i>	4P1 (HD395)	BGSC
GBJ 54	<i>Bt dakota</i>	4R1 (HD511)	BGSC
GBJ 55	<i>Bt indiana</i>	4S2 (HD521)	BGSC
GBJ 56	<i>Bt wuhanensis</i>	4T1 (HD525)	BGSC
GBJ 57	<i>Bt kysushensis</i>	4U1 (HD541)	BGSC
GBJ 58	<i>Bt tochiensis</i>	4Y1 (HD868)	BGSC
GBJ 89	<i>Bt mexicanensis</i>	4AC1 (GM43)	BGSC
GBJ 90	<i>Bt toguchini</i>	4AD1	BGSC
GBJ 91	<i>Bt amagiensis</i>	4AE1	BGSC
Bt 350	<i>Bt berliner</i>	DSMZ	Origin unknown
Bt 2040	<i>Bt berliner</i>	DSMZ	
Bt 2046	<i>Bt berliner</i>	ATCC 10792	Mediterranean flour moth
KRA 40	<i>B. mycoides</i>		
KRA 41	<i>B. mycoides</i>		
AND 1326	<i>B. mycoides</i>	KNC 3-15	J. Mahillon
KNC 1-2	<i>B. mycoides</i>		Bell & Friedman (1994)
KNC 2-13	<i>B. mycoides</i>	7A2 (ATCC19213)	Bell & Friedman (1994)
KNC 2-18	<i>B. mycoides</i>		Bell & Friedman (1994)
KNC 3-1	<i>B. mycoides</i>		Bell & Friedman (1994)
MYC 001	<i>B. mycoides</i>		IEBC
MYC 003	<i>B. mycoides</i>		IEBC
MYC 005	<i>B. mycoides</i>		IEBC
MYC 299	<i>B. mycoides</i>		DSMZ
MYC 650	<i>B. mycoides</i>		DSMZ
BCSL108	<i>B. mycoides</i>		Activated sludge
BCSL109	<i>B. mycoides</i>		Activated sludge
BCSL110	<i>B. mycoides</i>		Activated sludge
BCSL128	<i>B. mycoides</i>		Seine estuary
KBS 1-15	<i>B. mycoides</i>		J. Mahillon
KBS 1-16	<i>B. mycoides</i>		Bell & Friedman (1994)
KBS 1-4	<i>B. mycoides</i>		Bell & Friedman (1994)
KBS 2-12	<i>B. mycoides</i>		Bell & Friedman (1994)
NRRL B-347	<i>B. mycoides</i>		J. Mahillon
NRRL B-3436	<i>B. mycoides</i>		J. Mahillon
NRRL NRS306	<i>B. mycoides</i>		J. Mahillon
NRRL NRS371	<i>B. mycoides</i>		J. Mahillon



NRRL NRS1216	<i>B. mycoides</i>	J. Mahillon
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DMU/NERI: National Environmental Research Institute, Denmark

DSMZ: Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH  
(German Collection of Microorganisms and Cell Cultures)

5 IEBC: International Entomopathogenic Bacillus Centre (WHO), Institute Pasteur, France

BGSC: Bacillus Genetic Stock Center, the Ohio State University, USA

## 10 Other Embodiments

It is to be understood that while the invention has been described in conjunction with the detailed description thereof, the foregoing description is intended to illustrate and not limit the scope of the invention, which is defined by the scope of the appended claims. Other aspects, advantages, and modifications are within the scope of the following claims.

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## SEQUENCE LISTING

### GENERAL INFORMATION:

5           NUMBER OF SEQUENCES: 10

### INFORMATION FOR SEQ ID NO:1:

#### SEQUENCE CHARACTERISTICS:

- 10           • LENGTH: 20 base pairs
- TYPE: nucleic acid
- STRANDNESS: single
- TOPOLOGY: linear

15           SEQUENCE DESCRIPTION FOR SEQ ID NO:1:  
             5'-GGC GAA ACA TGA CGA AAA AC-3'

### INFORMATION FOR SEQ ID NO:2:

#### SEQUENCE CHARACTERISTICS:

- 20           • LENGTH: 25 base pairs
- TYPE: nucleic acid
- STRANDNESS: single
- TOPOLOGY: linear

25           SEQUENCE DESCRIPTION FOR SEQ ID NO:2:  
             5'-CCT CGT TAT GTA GCA ATC GTA TTA C-3'

### INFORMATION FOR SEQ ID NO:3:

#### SEQUENCE CHARACTERISTICS:

- 30           • LENGTH: 17 base pairs
- TYPE: nucleic acid
- STRANDNESS: single
- 35           • TOPOLOGY: linear

             SEQUENCE DESCRIPTION FOR SEQ ID NO:3:  
             5'-TTA CGT GAC GTC CCA TC-3'

INFORMATION FOR SEQ ID NO:4:

SEQUENCE CHARACTERISTICS:

- LENGTH: 17 base pairs
- TYPE: nucleic acid
- STRANDNESS: single
- TOPOLOGY: linear

SEQUENCE DESCRIPTION FOR SEQ ID NO:4:

5'-TGC GAC ATG GGT ACA AC-3'

INFORMATION FOR SEQ ID NO:5:

SEQUENCE CHARACTERISTICS:

- LENGTH: 21 base pairs
- TYPE: nucleic acid
- STRANDNESS: single
- TOPOLOGY: linear

SEQUENCE DESCRIPTION FOR SEQ ID NO:5:

5'-CAA CCA TCG TCA TCG TCA ATT-3'

INFORMATION FOR SEQ ID NO:6:

SEQUENCE CHARACTERISTICS:

- LENGTH: 28 base pairs
- TYPE: nucleic acid
- STRANDNESS: single
- TOPOLOGY: linear

SEQUENCE DESCRIPTION FOR SEQ ID NO:6:

5'-AAA AGG TAA CAA ATT ACT TAG TTG ATG G-3'

INFORMATION FOR SEQ ID NO:7:

SEQUENCE CHARACTERISTICS:

- LENGTH: 27 base pairs
- TYPE: nucleic acid
- STRANDNESS: single
- TOPOLOGY: linear

SEQUENCE DESCRIPTION FOR SEQ ID NO:7:

5'-CGA AGT TAA ATT ACT CCC TTC TTC CTT-3'

INFORMATION FOR SEQ ID NO:8:

SEQUENCE CHARACTERISTICS:

- LENGTH: 20 base pairs
- TYPE: nucleic acid
- STRANDNESS: single
- TOPOLOGY: linear

SEQUENCE DESCRIPTION FOR SEQ ID NO:8:  
5'-GGG TTA TAT GTT CCA GAA TC-3'

INFORMATION FOR SEQ ID NO:9:

SEQUENCE CHARACTERISTICS:

- LENGTH: 22 base pairs
- TYPE: nucleic acid
- STRANDNESS: single
- TOPOLOGY: linear

SEQUENCE DESCRIPTION FOR SEQ ID NO:9:  
5'-GTA ACT AAA TCA GAT TGG TTC T-3'

INFORMATION FOR SEQ ID NO:10:

SEQUENCE CHARACTERISTICS:

- LENGTH: 26 base pairs
- TYPE: nucleic acid
- STRANDNESS: single
- TOPOLOGY: linear

SEQUENCE DESCRIPTION FOR SEQ ID NO:10:  
5'-GAC CTT CAA AAG GTG TAG AAT TAA GG-3'

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## CLAIMS

1. A method for detecting the presence or absence of *Bacillus anthracis* in a biological or clinical sample from an individual or in a non-biological sample, said method comprising:
- 5 performing at least one cycling step, wherein said cycling step comprises an amplifying step and a hybridizing step, wherein said amplifying step comprises contacting said sample with a pair of capA primers to produce a capA amplification product if a *B. anthracis* capA nucleic acid molecule is present in said sample. Mentioned capA amplification product is being amplified further by performing at least one additional cycling step, comprising contacting said sample
- 10 and said capA amplification product with a pair of capA nested primers, to produce a nested capA amplification product. Mentioned hybridizing step of said method comprises contacting said sample with a capA probe, wherein the capA probe is labeled with a detectable label and detecting the presence or absence of released labeling signal, wherein the presence of released labeling signal is indicative of the presence of *B. anthracis* in said sample, and wherein the
- 15 absence of released labeling signal is indicative of the absence of *B. anthracis* in said sample.
2. The method of claim 1, wherein said pair of capA primers comprises a first capA primer and a second capA primer, wherein said first capA primer comprises the nucleotide sequence 5'-GGC GAA ACA TGA CGA AAA AC-3' (SEQ ID NO:1), and wherein said second capA primer
- 20 comprises the sequence 5'-CCT CGT TAT GTA GCA ATC GTA TTA C-3' (SEQ ID NO:2) or contiguous nucleotides hereof.
3. The method of claim 1, wherein said pair of nested capA primers further comprises a third capA primer and a fourth capA primer, wherein said third nested capA primer comprises the
- 25 sequence 5'-TTA CGT GAC GTC CCA TC-3' (SEQ ID NO:3), and wherein said fourth nested capA primer comprises the sequence 5'-TGC GAC ATG GGT ACA AC-3' (SEQ ID NO:4) or contiguous nucleotides hereof.
4. The method of claim 1, wherein said first capA probe comprises the sequence 5'-CAA CCA
- 30 TCG TCA TCG TCA ATT-3' (SEQ ID NO:5) or contiguous nucleotides hereof.
5. The method of claim 1, wherein said detection comprises quantitation by means of said FRET or said probe-specific voltage peak (PSVP).
- 35 6. The method of claim 1, wherein said detecting step is performed after each (amplification?) cycling step.

7. The method of claim 1, wherein said detecting step is performed in real time.

8. The method of claim 1, wherein the presence of said FRET detection signal within 100 cycling steps is indicative of the presence of *B. anthracis*.

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9. The method of claim 1, wherein the presence of said FRET detection signal within 50 cycling steps is indicative of the presence of *B. anthracis*.

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10. The method of claim 1, wherein the presence of said FRET detection signal within 30 cycling steps is indicative of the presence of *B. anthracis*.

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11. The method of claim 1, wherein said biological sample is derived from the group consisting of dermal swabs, nasal swabs, cerebrospinal fluid, blood, sputum, bronchio-alveolar lavage, bronchial aspirates, and feces.

12. The method of claim 1, wherein said non-biological sample is selected from the group consisting of powders, filtered air samples, surface swipes, dust, dirt and soil samples and rinse products from solid materials.

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13. The method of claim 1, further comprising: performing at least one cycling step, wherein a cycling step comprises an amplifying step and a hybridizing step, wherein said amplifying step comprises contacting said sample with a pair of Lef primers to produce a Lef amplification product if a *B. anthracis* Lef nucleic acid molecule is present in said sample. The Lef amplification product being further amplified by performing at least one additional cycling step comprising contacting said sample and said Lef amplification product with a pair of Lef nested primers to produce a nested Lef amplification product. The hybridizing step of said method comprises contacting said sample with a Lef probe, wherein the Lef probe is labeled with a detectable label and detecting the presence or absence of released labeling signal, wherein the presence of released labeling signal is indicative of the presence of *B. anthracis* in said sample, and wherein the absence of released labeling signal is indicative of the absence of *B. anthracis* in said sample.

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14. The method of claim 13, wherein said pair of Lef primers comprises a first Lef primer and a second Lef primer, wherein said first Lef primer comprises the sequence 5'-AAA AGG TAA CAA ATT ACT TAG TTG ATG G-3' (SEQ ID NO:6), and wherein said second Lef primer comprises the sequence 5'-CGA AGT TAA ATT ACT CCC TTC TTC CTT-3' (SEQ ID NO:7) or contiguous nucleotides hereof.

15. The method of claim 13, wherein said pair of nested Lef primers comprises a third Lef primer and a fourth Lef primer, wherein said third Lef primer comprises the sequence 5'-GGG TTA TAT GTT CCA GAA TC-3' (SEQ ID NO:8), and wherein said fourth Lef primer comprises the sequence 5'-GTA ACT AAA TCA GAT TGG TTC T-3' (SEQ ID NO:9) or contiguous nucleotides hereof.

16. The method of claim 13, wherein said first Lef probe comprises the sequence 5'-GAC CTT CAA AAG GTG TAG AAT TAA GG-3' (SEQ ID NO:10) or contiguous nucleotides hereof.

17. The method of claim 1, wherein said cycling step is performed on a control sample.

18. The method of claim 17, wherein said control sample comprises said portion of said *B. anthracis* capA nucleic acid molecule.

19. The method of claim 1, wherein said cycling step uses a pair of control primers and a control probe, wherein said control primers and said control probe are other than said capA primers and capA probe, wherein said amplifying step produces a control amplification product, wherein said control probes hybridize to said control amplification product.

20. A method for detecting the presence or absence of *B. anthracis* in a biological sample from an individual or in a non-biological sample, said method comprising: performing at least one cycling step, wherein a cycling step comprises an amplifying step and a hybridizing step, wherein said amplifying step comprises contacting said sample with a pair of capA primers to produce a capA amplification product if a *B. anthracis* capA nucleic acid molecule is present in said sample. The capA amplification product being further amplified by performing at least one additional cycling step comprising contacting said sample and said capA amplification product with a pair of capA nested primers to produce a nested capA amplification product. The hybridizing step of said method comprises contacting said sample with a capA probe, wherein the capA probe is labeled with a donor fluorescent moiety and a corresponding acceptor fluorescent moiety; and detecting the presence or absence of Förster Resonance Energy Transfer (FRET) between said donor fluorescent moiety and said acceptor fluorescent moiety of said capA probe, wherein the presence of FRET is indicative of the presence of *B. anthracis* in said sample, and wherein the absence of FRET is indicative of the absence of *B. anthracis* in said sample.

21. A method for detecting the presence or absence of *B. anthracis* in a biological sample from an individual or in a non-biological sample, said method comprising: performing at least one cycling step, wherein a cycling step comprises an amplifying step and a hybridizing step, wherein said amplifying step comprises contacting said sample with a pair of Lef primers to  
5 produce a Lef amplification product if a *B. anthracis* Lef nucleic acid molecule is present in said sample. The Lef amplification product being further amplified by performing at least one additional cycling step comprising contacting said sample and said Lef amplification product with a pair of Lef nested primers to produce a nested Lef amplification product. The hybridizing step of said method comprises contacting said sample with a Lef probe, wherein the Lef probe  
10 is labeled with a donor fluorescent moiety and a corresponding acceptor fluorescent moiety; and detecting the presence or absence of Förster Resonance Energy Transfer (FRET) between said donor fluorescent moiety and said acceptor fluorescent moiety of said Lef probe, wherein the presence of FRET is indicative of the presence of *B. anthracis* in said sample, and wherein the absence of FRET is indicative of the absence of *B. anthracis* in said sample.

22. An article of manufacture, comprising: a pair of capA primers; a pair of nested capA primers; a capA probe; and a donor fluorescent moiety and a corresponding acceptor fluorescent moiety.

23. The article of manufacture of claim 22, wherein said pair of capA primers comprises a first capA primer and a second capA primer, wherein said first capA primer comprises the sequence 5'-GGC GAA ACA TGA CGA AAA AC-3' (SEQ ID NO:1), and wherein said second capA primer comprises the sequence 5'-CCT CGT TAT GTA GCA ATC GTA TTA C-3' (SEQ ID NO:2) or contiguous nucleotides hereof.

24. The article of manufacture of claim 22, wherein said pair of nested capA primers further comprises a third capA primer and a fourth capA primer, wherein said third nested capA primer comprises the sequence 5'-TTA CGT GAC GTC CCA TC-3' (SEQ ID NO:3), and wherein said fourth nested capA primer comprises the sequence 5'-TGC GAC ATG GGT ACA AC-3' (SEQ ID  
30 NO:4) or contiguous nucleotides hereof.

25. The article of manufacture of claim 22, wherein said first capA probe comprises the sequence 5'-CAA CCA TCG TCA TCG TCA ATT-3' (SEQ ID NO:5) or contiguous nucleotides hereof.

26. The article of manufacture of claim 22, wherein said capA probe is labeled with said donor fluorescent moiety and said corresponding acceptor fluorescent moiety.



27. The article of manufacture of claim 22, further comprising a package insert having instructions thereon for using said pair of capA primers, said pair of nested capA primers, and said capA probe to detect the presence or absence of *B. anthracis* in a sample.

28. An article of manufacture, comprising: a pair of Lef primers; a pair of nested Lef primers; a Lef probe; and a donor fluorescent moiety and a corresponding acceptor fluorescent moiety.

29. The article of manufacture of claim 28, wherein said pair of Lef primers comprises a first Lef primer and a second Lef primer, wherein said first Lef primer comprises the sequence 5'-AAA AGG TAA CAA ATT ACT TAG TTG ATG G-3' (SEQ ID NO:6), and wherein said second Lef primer comprises the sequence 5'-CGA AGT TAA ATT ACT CCC TTC TTC CTT-3' (SEQ ID NO:7) or contiguous nucleotides hereof.

30. The article of manufacture of claim 28, wherein said pair of nested Lef primers comprises a third Lef primer and a fourth Lef primer, wherein said third Lef primer comprises the sequence 5'-GGG TTA TAT GTT CCA GAA TC-3' (SEQ ID NO:8), and wherein said fourth Lef primer comprises the sequence 5'-GTA ACT AAA TCA GAT TGG TTC T-3' (SEQ ID NO:9) or contiguous nucleotides hereof.

31. The article of manufacture of claim 28, wherein said first Lef probe comprises the sequence 5'-GAC CTT CAA AAG GTG TAG AAT TAA GG-3' (SEQ ID NO:10) or contiguous nucleotides hereof.

32. A method for detecting the presence or absence of *B. anthracis* in a biological sample from an individual or in a non-biological sample, said method comprising: performing at least one cycling step, wherein a cycling step comprises an amplifying step and a hybridizing step, and a dye-binding step wherein said amplifying step comprises contacting said sample with a pair of capA primers to produce a capA amplification product if a *B. anthracis* capA nucleic acid molecule is present in said sample. The capA amplification product being further amplified by performing at least one additional cycling step comprising contacting said sample and said capA amplification product with a pair of capA nested primers to produce a nested capA amplification product, wherein said dye-binding step comprises contacting said capA amplification product with a double-stranded DNA binding dye; and detecting the presence or absence of binding of said double-stranded DNA binding dye into said amplification product, wherein the presence of binding is indicative of the presence of *B. anthracis* in said sample, and wherein the absence of binding is indicative of the absence of *B. anthracis* in said sample.

33. A method for detecting the presence or absence of *B. anthracis* in a biological sample from an individual or in a non-biological sample, said method comprising: performing at least one cycling step, wherein a cycling step comprises an amplifying step and a hybridizing step, and a dye-binding step wherein said amplifying step comprises contacting said sample with a pair of Lef primers to produce a Lef amplification product if a *B. anthracis* Lef nucleic acid molecule is present in said sample. The Lef amplification product being further amplified by performing at least one additional cycling step comprising contacting said sample and said Lef amplification product with a pair of Lef nested primers to produce a nested Lef amplification product, wherein said dye-binding step comprises contacting said Lef amplification product with a double-stranded DNA binding dye; and detecting the presence or absence of binding of said double-stranded DNA binding dye into said amplification product, wherein the presence of binding is indicative of the presence of *B. anthracis* in said sample, and wherein the absence of binding is indicative of the absence of *B. anthracis* in said sample.

34. The method of claims 32 and 33, wherein said double-stranded DNA binding dye is selected from the group consisting of SYBRGreen I®, SYBRGold®, and ethidium bromide.

35. The method of claims 32 and 33, further comprising determining the melting temperature between said capA amplification product and said double-stranded DNA binding dye, wherein said melting temperature confirms said presence or absence of said *B. anthracis*.